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Original Article

In vitro, antioxidant activities of aqueous and methanol roselle (*Hibiscus sabdariffa*) calyces extracts from two localities in Cameroon

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Abstract

Background: Fighting against malnutrition and diseases such as oxidative stress diseases via a food-based approach could be achieved through identification, valorization, and promotion of local foods rich in macro- and micronutrients and phytochemical components. **Aim:** This study aimed to investigate the effect of agro-ecological conditions on the antioxidant capacity of the *Hibiscus sabdariffa* aqueous and methanol calyces extracts. **Material and methods:** The total phenolic content, the free radical DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl), and ferric reducing antioxidant power (FRAP) activities were evaluated in aqueous and methanol *Hibiscus sabdariffa* calyces extract samples from two localities (Dschang [western highland zone] and Ngaoundéré [high Guinean savanna zone]) in Cameroon. **Results:** The results obtained showed that these extracts contain an important amount of total phenolic compounds with no significant difference (P>0.05) between aqueous and methanol extracts and also between the origins of calyces. The extracts showed their ability to reduce Fe^{3+} to Fe^{2+} as well as their ability to reduce the free radical, DPPH$^{\bullet}$. Concerning the FRAP results, at the concentration of 12.5µg/mL, the absorbance of AEN (Aqueous Extract from Ngaoundéré locality) recorded the value (0.33±0.05) meanwhile at a far higher concentration of 200 µg/mL, the absorbance of MEN (Methanol Extract from Ngaoundéré locality) was the highest (1.39±0.06). The CI_{50} of methanol extracts was significantly low (P<0.05) compared to that of aqueous extracts. The value ranges were 11.31±0.15, 14.69±0.84, 18.07±0.63, 20.50±0.54, and 21.50±0.54 µg/mL for ascorbic acid, MEN (Methanol Extract from Ngaoundéré locality), MED (Methanol Extract from Dschang locality), AEN (Aqueous Extract from Ngaoundéré locality), and AED (Aqueous Extract from Dschang locality) respectively. **Conclusion:** These findings show that the antioxidant activity of methanol extract is higher than that of aqueous extract. *Hibiscus* calyces from the two localities could be used as a natural antioxidant in the treatment of oxidative stress diseases.

Keywords: Roselle calyces, aqueous and methanol extracts, antioxidant capacity.

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1 Introduction

Plant products are materials commonly used by the population in most developing countries, particularly those living in rural areas. *Hibiscus sabdariffa* L. is one of these plants and could even be considered as a functional food given some of its properties already studied. Hibiscus calyces possess some medicinal properties. They are used as laxatives and diuretics [1]. They have antihypertensive, cardioprotective [2,3], and hypocholesterolemic properties [4]. They also possess Hepatoprotective and anti-inflammatory properties [5,6]. The *Hibiscus sabdariffa* calyces possess high levels of organic acids, vitamin C, phytochemicals such as anthocyanins and phenols [7]. These calyces are used in the preparation of tonic and alcohol-free beverages (sorrel juice) and the production of jams and jellies. Calyces are also used to make an alcoholic fermented beverage that is similar to wine [8-10]. The juice extracted from the calyces of *Hibiscus sabdariffa* is commonly called "foléré" in Cameroon. The preparation of this sorrel juice is still done at the artisanal and traditional stage in households. It is very easy and simple because it does not require a specific technique and material. The calyces are boiled, usually adding other plants such as pineapple peelings and or pineapple juice, lemongrass leaves, avocado leaves, and synthetic aroma. At the end of the decoction and after filtration, sugar is most often added to decrease the sour taste of the juice. Previous works with this plant showed that consumption of "foléré" causes a significant increase in red blood cells and their indices and a significant decrease in total cholesterol and LDL cholesterol in humans [11,12]. What could be the influence of the harvesting/cultivated site and the nature of the extract on the antioxidant capacities of *Hibiscus sabdariffa* calyces in Cameroon? Data on the nutritional quality of *Hibiscus* calyces are needed for its recommendation as a functional food. Despite the increasing use or consumption of roselle juice in dieting in Cameroon, some of its pharmacological properties remain unknown. *H. sabdariffa* is a locally grown plant that could provide nutrients to a broad section of the population for long without requiring radical changes in their eating habits.

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According to Mady *et al.* [1], the nutritional value of this plant is strongly influenced by climatic and agro-ecological conditions. Cameroon is a country where climatic and ecological conditions make it possible to fragment the country into four agro-ecological zones [13]. Hence, the aims of this study were to quantify the total phenolic content and evaluate the antioxidant capacity of methanol and aqueous roselle extracts. Concomitantly, the possible influence of the cultivated site on Hibiscus antioxidant properties was also assessed. Information on the antioxidant capacity of roselle calyces could contribute to a better fight against the diseases linked to oxidative stress such as cardiovascular diseases, cancers, neurodegenerative diseases, etc.

2 Material and Methods

2.1 Plant material

Roselle (*Hibiscus sabdariffa*) dried calyces were collected from farmers in Dschang and Ngaoundéré localities situated in western highland and high Guinean savanna agro-ecological zones respectively. Sun-dried calyces collected from each area were placed in polyethylene bags and transported to the laboratory for further treatments. The plant was identified at the National Herbarium of Cameroon where a voucher specimen was deposited under reference number 25776/SRF/Cam.

2.2 Aqueous and methanol extracts preparation

The aqueous extracts called AEN (Aqueous Extract from Ngaoundéré locality) and AED (Aqueous Extract from Dschang) were prepared according to the method described by Maffo *et al.* [11]. Briefly, the aqueous extract was obtained via the decoction procedure and the inputs were in the proportion 21.5 g of calyces/1 liter tap water. Concerning the methanol extract called MEN (Methanol extract from Ngaoundéré locality) and MED (Methanol Extract from Dschang locality), the calyces were crushed into a fine powder and 100 g of the powder was infused in 400 mL of methanol with two times daily shaking during 48 hours. All solutions obtained were then filtered with Whatman paper filter No 1. Finally, the water contained in the aqueous extract was evaporated until a solid extract was obtained. The filtrate was distributed in sufficient quantities in wide-bottom jars and placed in an oven at 45°C for two days. The methanol contained in the methanol extract was evaporated under reduced pressure at 63°C using a rotary evaporator (BUCHI Rotavapor R-200).

2.3 Total phenolic compounds determination

The total phenolic compounds were determined by the spectrophotometric method using the Folin Ciocalteu reagent as described by Gao *et al.* [14]. Briefly, 0.01 mL of each extract solution (5 mg/mL), 1.39 mL of distilled water, and 0.2 mL of Folin Ciocalteu reagent were introduced in each test tube. The mixture was left to stand at room temperature for 3 minutes and thereafter, 0.4 mL of sodium carbonate solution (Na_2CO_3, 20%) was added in each tube, mixed, and placed in a water bath at 40°C for 20 minutes. The absorbance was measured at 760 nm using a UV–visible spectrophotometer (GENESYS 20, Model 4001/4). The total phenolic content was expressed as milligrams gallic acid equivalents (mgGAE)/gram of plant material using a regression equation and a gallic acid calibration curve (R^2=0.931).

2.4 Measurement of DPPH radical-scavenging activity

Free radical scavenging was evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method stated by Mensor *et al.* [15]. The mother solutions of extract were prepared by dissolving 2 mg of each extract in 1 mL of methanol. 100 µL of extract (2000 µg/mL) was introduced in the tubes of two first lines. After, 100 µL of methanol was introduced in all tubes from the second line followed by the two-fold serial dilution. Finally, 900 µL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) methanol solution (20mg/mL) was added in the first columns three and 900 µL of methanol in the last column to give a final extract concentration value of 12.5, 25, 50, 100, and 200 µg/mL. After 30 min of incubation in the dark at room temperature, the optical densities were measured at 517 nm. The percentage of free radical-scavenging activity was calculated, based on the following equation: Free radical-scavenging activity (%) = [(Absorbance of DPPH - Absorbance of the sample) / Absorbance of DPPH- Absorbance of control] x 100. Ascorbic acid was used as control and each assay was done in triplicate.

The IC_{50}, being the smallest concentration of the antioxidants necessary for 50% of inhibition, was calculated using the linear regression equation curve (R^2 = 0.9976) obtained by plotting the radical scavenging percentages against the logarithmic values of the concentration of test samples.

2.5 Measurement of Ferric Reducing Antioxidant Power (FRAP) activity

The antioxidant activity of roselle extract was also evaluated using the ferric reducing power assay described by Padmaja *et al.*[16] with some modifications. 200, 100, 50, 25 and 12,5 µL of each aqueous and methanol extract solutions (2090 µg/mL) were mixed with 500 µL of phosphate buffer (0.2 M, pH 6.6) and 500 µL of 1% potassium ferricyanide and incubated at 50°C for 20 min. Thereafter, 500 µL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. 500 µL supernatant was diluted with 500 µL of water and mixed with 100 µL of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. Increased absorbance of the reaction mixture indicates a higher reduction capacity of the extracts.

2.6 Statistical analyses

Results were reported as means ± SD and statistical analyses were done using Graph pad prism version 5.00 software. The

following statistical tests were performed: one-way ANOVA and Newman-Keuls. A P-value of less than 0.05 was considered significant.

3 Results

3.1 Total phenolic content of *H. sabdariffa* calyces extracts

The total phenolic content of the roselle calyces harvested in two agro-ecological areas in Cameroon is presented in figure 1. The figure shows that there was no significant difference between the total phenolic content of different extracts. However, the methanol extracts presented the highest total concentration of phenolic compounds.

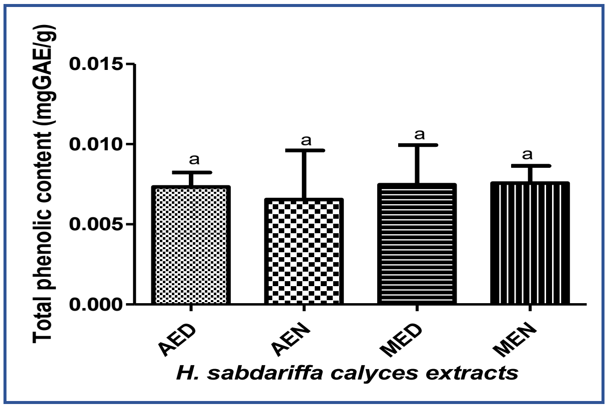


Figure 1: Total phenolic content of aqueous and methanol roselle (Hibiscus sabdariffa) calyces extracts
Values are means ± SD (standard deviation). For each extract, the histograms carrying the same letters are not significantly different at 5% probability, AED= aqueous extract from Dschang locality, AEN= Aqueous extract from Ngaoundéré locality, MED= methanol extract from Dschang locality, MEN= methanol extract from Ngaoundéré locality, mgGAE/g= mg gallic acid equivalents per gram of plant material.

3.2 DPPH radical-scavenging activity of *H. sabdariffa* calyces extracts

Table 1 shows that the DPPH radical scavenging activity of the reference antioxidant (ascorbic acid) was significantly higher (P<0.05) than those of extracts at the concentrations 12.5; 25 and 50 µg/mL. However, at the concentration of 100 µg/mL, there was no significant difference between the ascorbic acid and the extract MEN (methanol extract from Ngaoundéré locality). Nevertheless, at the concentration 200 µg/mL, the inhibition percentages of all extracts [MEN (methanol extract from Ngaoundéré locality), MED (methanol extract from Dschang locality), AEN (aqueous extract from Ngaoundéré locality) and AED (aqueous extract from Dschang locality)] were significantly higher than the inhibition percentage of the ascorbic acid (reference antioxidant).

The results of figure 2 show that the methanol extracts demonstrated the best IC_{50} comparatively to aqueous extracts. Summarily, at the concentration 12.5 µg/mL, all extracts inhibited less than 50% of DPPH•; at the concentration, 25 µg/mL only the methanol extracts inhibited more than 50 % of DPPH•; but at the concentrations of 50, 100, and 200 µg/mL, all extracts inhibited more than 50 % of free DPPH radicals.

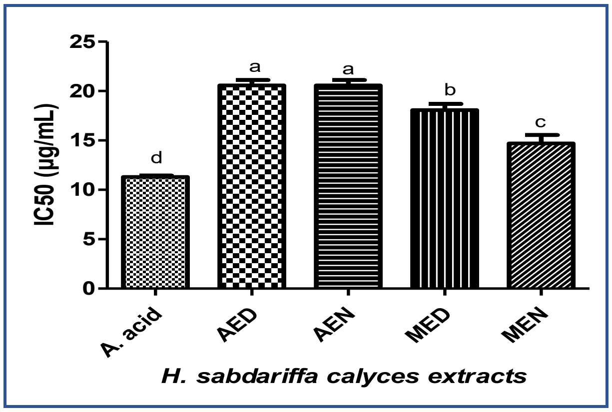


Figure 2: IC_{50} of aqueous and methanol roselle (Hibiscus sabdariffa) calyces extracts
Values are means ± SD (standard deviation). For each extract, the histograms carrying different letters are significantly different at 5% probability, AED= aqueous extract from Dschang locality, AEN= Aqueous extract from Ngaoundéré locality, MED= methanol extract from Dschang locality, MEN= methanol extract from Ngaoundéré locality. A. acid= ascorbic acid, IC_{50}= smallest concentration of the antioxidants necessary for 50% of inhibition.

3.3 Ferric Reducing Antioxidant Power (FRAP) activity of *H. sabdariffa* calyces extracts

The results of figure 3 show that the iron reduction capacity is proportional to the increased concentration of extracts. There was a significant difference (p<0.05) between the ascorbic acid activity and all extracts at different concentrations. Moreover, at concentrations 12.5, 25, and 50 µg/mL the ferric reducing antioxidant power of the AEN extract (aqueous extract from Ngaoundéré locality) was the highest while the AED extract (Aqueous extract from Dschang locality) has presented the lowest activity.

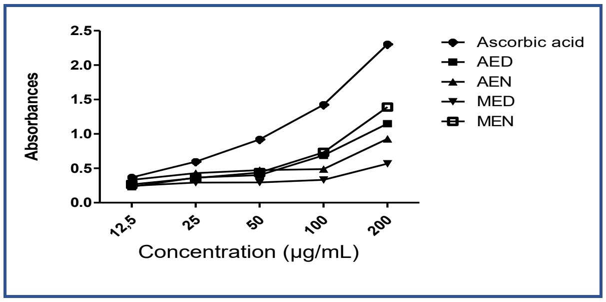


Figure 3: Antioxidant activity of aqueous and methanol roselle (*Hibiscus sabdariffa*) calyces extracts in FRAP (Ferric-ion Reducing Antioxidant Power) assay
AED= aqueous extract from Dschang locality, AEN= Aqueous extract from Ngaoundéré locality, MED= methanol extract from Dschang locality, MEN= methanol extract from Ngaoundéré locality

4 Discussion

The phytochemicals screening of aqueous and methanol extracts of Roselle calyces cultivated and harvested in two agro-ecological areas in Cameroon had revealed the presence of several classes of

Table 1: Antioxidant activity of aqueous and methanol roselle (Hibiscus sabdariffa) calyces extracts in DPPH free radical-scavenging assay

Concentrations (µg/mL)	Inhibition percentages of extracts and ascorbic acid				
	Ascorbic acid	AED	AEN	MED	MEN
12.5	72.30 ± 1.40[a]	36.54±1.57[b]	36.43±1.38[b]	36.09±2.55[b]	36.60±0.93[b]
25	83.81±1.17[a]	47.75±0.97[e]	47.46±1.28[d]	53.77±0.08[c]	57.83±1.78[b]
50	88.44±0.94[a]	62.72±1.02[c]	63.79±1.78[c]	64.86±0.33[c]	73.22±1.53[b]
100	88.55±0.95[a]	75.73±0.51[c]	72.97±1.55[c]	82.32±1.86[b]	87.92±2.34[a]
200	89.05±0.75[c]	94.93±1.03[b]	97.35±0.85[a]	95.55±1.28[a,b]	97.72±0.26[a]

Values are means ± SD (standard deviation). Values carrying different letters in the same column and the same row are significantly different at 5% probability, AED= aqueous extract from Dschang locality, AEN= Aqueous extract from Ngaoundéré locality, MED= methanol extract from Dschang locality, MEN= methanol extract from Ngaoundéré locality.

chemical compounds such as alkaloids, flavonoids, tannins, phenols, and anthocyanins but also the absence of compounds such as saponins, sterols, and triterpenoids [12]. These compounds are known for their positive effects on various physiological processes such as the germination of grains or the maturation of fruits. The regular consumption of foods rich in compounds that possess the antioxidant capacities (phytonutrients) is associated with a low risk of oxidative stress diseases and mortality prevalence [17]. Most of these phytochemical compounds are known for their positive effects on health and general wellbeing. Medicinal properties: antioxidant, antihypertensive, antibacterial, heart-protective, etc. [18-21] attributed to *Hibiscus sabdatiffa* calyces could be possible via these phytochemical compounds present in the plant.

The plant is the site of intense metabolic activity resulting in the synthesis of a wide variety of active ingredients. This metabolic process is linked to the very living conditions of the plant, which has to face multiple attacks from the environment in which it grows. It is therefore conceivable that the plant can develop a particular metabolism allowing it to synthesize certain secondary metabolites to defend itself [22]. The results of the quantification of the total phenolic compounds have shown no significant difference but the phenolic content of the sample harvested in the Adamaoua locality was slightly higher than that harvested in the Dschang locality. This can be explained by the fact that in Adamaoua locality, plants are more stressed during a long period of drought. It is known that during, stress, more phytochemicals are produced in the plant to withstand the conditions [23]. The Adamaoua locality situated in the Guinean savanna zone where one of the samples was collected is characterized by a mean annual rainfall of approximately 1500 mm with 150 days of rainfall per year while the Dschang locality is situated in the Western high plateau zone with 2000 to less than 4000 mm rainfall [13]. However, the total phenol content of the methanol extracts was higher compared to those of aqueous extracts. Phenolic compounds are very important components of the plant because they exert antioxidant activity by inactivating free radicals or preventing the decomposition of hydrogen peroxide into free radicals [24, 25]. The antiradical activity of the extracts was evaluated *in vitro* by the DPPH (1,1-diphenyl-2-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) tests.

Concerning the DPPH test, the methanol calyces' extracts from the Ngaoundéré locality (MEN) have the highest inhibition percentages. These results corroborate the results of the total phenolic content because the same extract (MEN) showed the highest total phenol content. Liuqing *et al.* [26] by studying the antioxidant capacity of *H. sabdariffa* calyces extracts found a positive correlation between total phenol content and antioxidant capacity. The ability of the extracts to reduce the level of $DPPH^{\bullet}$ would, therefore, be related to the presence of the phenolic compounds in the latter. Because of the results of the IC_{50s} (smallest concentration of the antioxidants necessary for 50% of inhibition), the extracts are powerful natural antioxidants, that is they possess neutralization capacities of the free radical $DPPH^{\bullet}$ since, at the concentration of 200 µg/mL, the extracts showed percent inhibition significantly higher than that of ascorbic acid (reference antioxidant). According to Souri *et al.* [27], the potential of the antioxidant activity of a plant extract is divided into three groups: high (IC_{50} <20 µg/mL), moderate (20 µg/mL <IC_{50} <75 µg/mL) and low (IC_{50}> 75 µg/mL). Given this, the methanol extracts of the roselle calyces have a high antioxidant potential whereas the aqueous extracts have a moderate antioxidant potential. The results of the FRAP showed that the reduction capacity was proportional to the increase in the concentration of the samples. The higher the absorbance readout, the better the extract with a good ferric reducing power. The presence of reducing agents such as antioxidants in the extracts could, therefore, allow the reduction of ferric iron to ferrous iron. Thus, the aqueous and methanol extracts of the *H. sabdariffa* calyces grown in Cameroon can reduce Fe^{3+} to Fe^{2+}.

5 Conclusion

The aqueous and methanol extracts of *Hibiscus sabdariffa* calyces, cultivated and harvested in these two different agro-ecological areas in Cameroon, have shown their ability to reduce DPPH free radicals and to reduce ferric iron to ferrous iron. The calyces harvested in Adamaoua locality (Guinean savanna zone) and the methanol extracts have shown a slightly higher antioxidant activity compared to that of calyces harvested in Dschang locality (Western high plateau zone) and the aqueous extracts.

Hibiscus sabdariffa calyces can, therefore, be used as a source of natural antioxidants to fight against diseases related to oxidative stress since these natural antioxidants present a minimal risk of side effects compared to synthetic antioxidants.

Author contribution: Dr. Maffo conceived and designed the study, and undertook the literature research. He also performed the data analysis, carried out the statistical analysis, and drafted the manuscript. All authors participated in the experiment and data acquisition. Dr. Agbor and Prof Gouado reviewed the manuscript. All authors approved the final version before submission. All authors have read and agreed to the published version of the manuscript.

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